THE CULTURE OF BRINE ALGAE [1]

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The biological productivity of the solar evaporation ponds used in salt manufacture is apparent even to the casual observer: they are a deep green or rich red. The ecological association in these ponds was the subject of several investigations (Peirce, 1914; Baas-Becking, 1928; Carpelan, 1953). Carpelan concluded that the productivity of the ponds, per unit area, was comparable to that of the ocean in its richer spots. But the ponds, being only half a meter deep, have a much more concentrated plankton community than does the productive zone of the ocean, which is 10 to 15 meters deep.

With the increased interest in recent years in mass culturing of algae for food production, the possibility of utilizing these evaporation ponds for such purposes has been considered. With this in mind, a laboratory study was undertaken on the cultivation of several of the unicellular algae which thrive in the ponds.

Pure cultures were isolated by the procedures described by Pringsheim (1946) with the added advantage of utilizing antibiotics, as suggested by Spencer (1952). The following five algae were studied:

Stichococcus sp.
Platymonas sp.
Dunaliella viridis
Dunaliella salina
Stephanoptera gracilis

These were isolated from the ponds of the Leslie Salt Company, on San Francisco Bay. The author is indebted to this company for many privileges during the course of the study.

SALINITY TOLERANCE

The determination of the range of salinity tolerated by the different algae under study is of primary importance. For this purpose several strains of the algae were inoculated into solutions of artificial sea water of varying concentrations.

Stichococcus strains numbers 1, 2 and 3, *Platymonas* strains 5 and 7, *Dunaliella viridis* strains 6, 8 and 9, *Dunaliella salina* strain 10, and *Stephanoptera gracilis* were studied. The strain numbers refer to the evaporation ponds from which they were isolated. Pond number 1 contained San Francisco Bay water (concentration less than sea water). The successive ponds contained increasing concentrations of brine to NaCl saturation in pond number 10.

Artificial sea water was prepared after Pringsheim (1946). Multiples of the sea water formula were used for preparing the desired concentrations. Concentra-

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tions up to ten-fold sea water were prepared; the solutions of concentration of X 5 and above were saturated with respect to the $CaSO_4$, excess solid being filtered off. Dilutions of sea water to ½, ⅒, as well as fresh water, were also used. All solutions were equally enriched with 50 mg. KNO_3, 10 mg. KH_2PO_4 and Hutner's trace elements mixture (Hutner *et al.*, 1950). Five-ml. portions of the various solutions were pipetted into test tubes and equally inoculated. The test tubes were kept in an inclined position under continuous illumination. Light was provided by several fluorescent lights suspended above the test tubes; its intensity was about 150 foot candles.

Growth was estimated by cell counts after 16 days of culture. At this time the tubes in which no growth had taken place were re-inoculated with 0.1 ml. of the culture which was nearest to them in concentration. Thus, if a culture in the tubes of X 7 sea water did not grow, it was inoculated with the growing X 6 culture. The results of these experiments are given in Table I. The results are expressed as per cent of the growth in sea water, except for the values for *Stephanoptera gracilis,* which are expressed as per cent age of X 2 sea water medium. The results of the secondary inoculation are expressed as T (for trainable) and NT (for not trainable).

TABLE I

Relative growth of algal strains isolated from different salinities in media of different concentrations

Strain:	Medium Concentrations												
	0	1/10	1/2	1	2	3	4	5	6	7	8	9	10
Stichococcus sp.													
1.	30%	74%	170%	100%	NT								
2.	T	46%	135%	100%	15%	NT							
3.		T	90%	100%	40%	NT							
Platymonas sp.													
5.	77%	77%	90%	100%	90%	47%	NT						
7.		50%	100%	100%	125%	230%	60%	NT					
Dunaliella viridis													
6.	63%	82%	240%	100%	100%	95%	85%	50%	26%	NT			
8.	NT	34%	?	100%	225%	320%	230%	125%	100%	18%	T		
9.		T	60%	100%	210%	400%	320%	310%	120%	40%	T	T	T
Dunaliella salina													
10.		T	100%	100%	160%	220%	215%	185%	145%	80%	60%	55%	35%
Stephanoptera gracilis													
11.			T	12%	100%	120%	135%	67%	30%	T	T	T	

(Concentrations in multiples of sea water; T and NT refer to trainability or non-trainability of the algae for growth in the specific concentrations. Growth in sea water taken as base value for all strains except *Stephanoptera gracilis* in which × 2 sea water was taken as base value.)

From Table I it is apparent that the *Stichococcus* strains under study are primarily brackish water organisms. The *Platymonas* sp. appear to be more resistant to salt, although they too did not grow in the highly concentrated brines. The last three algae, all of the Polyblepharidaceae family, tolerate a wide range of salinity. Of these, *Stephanoptera gracilis* did not grow well at concentrations below X 2 sea water.

TEMPERATURE

To determine the temperature range for the organisms under study, the following procedure was adopted. Sea water was used for *Dunaliella viridis, Platy-*

monas sp. and *Stichococcus* sp.; *Dunaliella salina* and *Stephanoptera gracilis* were cultured in X 2 sea water. Media were enriched with nitrate, phosphate and Hutner's trace elements. Portions of sterile media were inoculated with the above mentioned cultures, five-ml. portions being pipetted into sterile test tubes.

Cultures were incubated in thermostatically controlled incubators, with light provided from two 15-watt fluorescent lamps. The test tubes were set on an inclined rack at a distance of 20 centimeters from the light source. Light intensity at the culture tube level was above 100 foot candles. After 10 days of continuous illumination, growth was estimated in the cultures by cell count. The results of the counts are given in Table II. The range of temperatures recorded by Carpelan (1953) for the evaporation ponds of the same region was from 8° to 20° in the winter, and 15° to 30° in the summer.

pH Effect

The effect of pH on the growth of the algae was investigated with the aid of media buffered with "Tris" (1, 3-propendiol-2-amino-2-hydroxymethyl). The pH range between 7 and 9 was investigated since this is the most common value for the sea water brines. Culture media were prepared as before except for the addition of 0.4 M "Tris" buffer; pH was adjusted to 7.2, 8.0 or 9.0. Five-ml. portions of the media were pipetted into test tubes and equally inoculated. The test tubes were kept inclined under continuous illumination. Cell counts were made after 9 days of growth. The results are recorded in Table III.

Table II

Effect of temperature on growth of brine algae

(Cells per cubic millimeter)

Temperature ° C.	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
8–10°	20	90	2,700	120	4,200
14–16°	200	560	36,000	340	24,000
24–26°	160	640		1,100	47,000
30°	450	1,800	35,000	1,000	40,000
35°	25	40	no growth	1,100	16,000

As seen from the table only *Stichococcus* sp. and *Platymonas* sp. show a definite preference for higher pH values. The other three algae do not seem to be very sensitive to pH changes in the range studied.

Phosphate Concentration

The optimum range of phosphate concentration for the algae under study was determined in an experiment in which 5 ml. of inoculated media in test tubes were enriched with graded quantities of phosphate. The media were buffered to pH 8 with .04 M "Tris" buffer. Nitrate and micronutrients were supplied as in the previous experiments. Cell counts were made after 10 days of cultivation under continuous illumination. The results are tabulated in Table IV. The optimum

TABLE III

Effect of pH on growth of brine algae

	Original pH	Final pH	Cells/mm.[3]
Dunaliella salina	7.2	7.5	770
	8.0	8.1	1,300
	9.0	9.1	1,380
Stephanoptera gracilis	7.2	7.8	1,100
	8.0	8.1	740
	9.0	9.2	760
Dunaliella viridis	7.2	7.6	24,000
	8.0	8.0	15,000
	9.0	9.2	20,000
Stichococcus sp.	7.2	7.4	16,000
	8.0	7.9	25,000
	9.0	9.2	69,000
Platymonas sp.	7.2	7.5	570
	8.0	8.0	1,000
	9.0	9.2	2,270

range as seen in the table is in the same range of concentration which is recommended for use with several other marine algae (Koch, 1953; Ketchum and Redfield, 1938; Kylin, 1943).

NITROGEN SUPPLY

The growth of the algae on nitrate or ammonium as the nitrogen source was investigated. These are easily available forms of fixed nitrogen for mass cultivation of plants.

Media were prepared as before, buffered with "Tris" to pH 8.1. Phosphate and trace elements were supplied. Potassium nitrate was added in concentrations of 50 mg./100 ml. NH_4Cl was added in concentrations of 26.5 mg./100 ml. Cell counts were done after two weeks of culture under continuous illumination. In Table V the results of this experiment are summarized.

TABLE IV

Effect of phosphate concentration on growth of brine algae

(Cells per cubic millimeter)

KH_2PO_4 (microgram/ml.)	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
0	340	280	1,300	270	8,500
20	450	630	3,000	520	8,800
40	710	600	2,800	830	12,700
100	540	450	2,400	700	13,700
200	420	300	1,400	730	13,500
500	200	190	1,200	560	12,300

In another experiment, summarized in Table VI, using an unbuffered medium and higher concentrations of nitrate and ammonium (½ mg. N/ml.), the superiority of nitrate over ammonium for growth of all the investigated algae was clearly demonstrated. In this experiment urea was also studied; it was found to be a superior nitrogen source only for *Dunaliella salina.*

The ability of the algae to grow when supplied with organic nitrogenous substances as sole nitrogen source was also investigated. Media were prepared enriched with phosphate and micro-nutrients. The nitrogen source was added to a final concentration of 50 micrograms N/ml. The media were buffered with "Tris" to either 7.5 or 9.0. Cell counts were made after 12 days of culture, the results being tabulated in Table VII.

TABLE V

Nitrate and ammonium as nitrogen sources for brine algae in media buffered to pH 8.1

(Cells per cubic millimeter)

	Dunaliella salina	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
NO_3	1,700	1,500	4,700	6,000	48,000
NH_4	1,900	2,500	13,000	6,300	28,000

Contrary to expectation, uric acid, which is the main nitrogen form in bird excrement, does not serve as a good nitrogen source for *Stephanoptera gracilis* although this alga is usually found to bloom in high tide pools on rocks which are coated with bird excrement. *Platymonas,* which also blooms under similar conditions, can utilize uric acid. *Platymonas* alone was found to grow on all the organic nitrogenous substances studied; this might be a clue to an observation that this alga appeared to coat glassware in which animal or plant materials are kept under running sea water in the laboratory. *Stichococcus* sp. prefers nitrate to uric acid and asparagin; this is in contrast to the strains studied by Ryther (1954) which apparently grow better on uric acid and asparagin than on nitrate.

TABLE VI

Effect of nitrogen source on growth of brine algae

(Number of cells per cubic millimeter and final pH values indicated)

Nitrogen source	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
	Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
NO_3	1,000	8.2	1,700	8.9	39,000	8.5	2,000	9.5	20,000	9.3
NH_4	600	6.4	700	5.0	6,000	5.5	400	6.5	10,000	6.9
Urea	1,400	7.8	500	7.5	6,000	7.5	300	8.3	8,000	7.8

The experiment was performed at two pH values with the hope of gaining information on the relative availability to the cells of the different ionic forms of the ex-

TABLE VII

Effect of various nitrogen sources on growth of brine algae

(Number of cells per cubic millimeter, and final pH values indicated)

Nitrogen source	Original pH	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
		Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
Nitrate	7.5	870	7.8	1,370	7.9	25,000	7.8	530	7.8	28,000	7.7
	9	1,700	9.0	380	8.9	39,000	9.1	1,100	9.1	70,000	9.1
Uric acid	7.5	880	7.5	0		3,000	7.5	530	7.6	11,000	7.6
	9	1,100	8.7	0		4,500	8.7	1,300	8.9	17,000	8.8
dl-Aspartic acid	7.5	0		0		0		130	7.3	0	
	9	0		0		0		200	8.6	0	
Glutamic acid	7.5	970	7.9	530	7.8	0		500	7.7	0	
	9	530	8.9	270	8.8	0		500	8.9	0	
Asparagine	7.5	210	7.6	120	7.6	0		470	7.7	8,000	
	9	210	8.6	110	8.7	0		1,200	8.9	8,000	
Glycine	7.5	0		0		0		1,000	7.7	0	
	9	0		0		0		1,200	8.9	0	

amined substances. The growth on uric acid was found in general to be pH-dependent in much the same way as growth on nitrate. On the other hand growth on glutamic acid seems to show a reverse pH response in the case of *Dunaliella salina*.

ORGANIC NUTRIENTS

The ability of the brine algae to grow in the dark on several organic substances, as well as the effect of these substances on the growth in light, was investigated. Two organic energy sources were investigated, glucose and acetate. The effect of

TABLE VIII

Effect of organic substances on growth of brine algae

(Cells per mm.^{3})

Medium	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
1. Mineral only	700	970	6,250	630	27,000
2. Glucose	670	980	11,300	430	26,000
3. Acetate	580	870	6,200	770	—
4. YE* Glucose	700	750	9,200	420	16,000
5. YE Acetate	780	720	7,200	970	—
6. BH** Glucose	1,300	1,070	11,500	700	20,000
7. BH Acetate	670	900	9,500	920	—

* Yeast extract.

** Brain-heart infusion.

these was studied alone and in conjunction with complex mixtures such as yeast extract (YE) or brain-heart infusion (BH). The media were enriched with nitrate, phosphate and trace elements, and strongly buffered to pH 8.1 with 0.1 *M* "Tris" buffer. Glucose or acetate were used to final concentration of ½%, brain-heart infusion to .02% and yeast extract to .01%. Five-ml. portions of the inoculated media were pipetted into test tubes. Two equal sets of test tubes were maintained, one under continuous illumination and the other in the dark. The illuminated tubes were counted after 7 days of culture. The results are given in Table VIII. No growth was detectable in the tubes which were kept in the dark for three weeks.

The inhibition of growth of *Stichococcus* by the acetate is remarkable. The inhibitory effect of undissociated acetate on *Chlorella* is discussed by Myers (1951). In our case, however, the concentration of undissociated acetate is very low (about .0003 *M*). *Dunaliella viridis* seems to be stimulated by glucose, while *Platymonas* is stimulated by acetate.

SUMMARY

1. The conditions which the brine algae require for growth were found to be relatively simple. The high temperatures in the pond waters during the summer are well within the tolerance range of these organisms.

2. Simple nitrogenous substances and no organic supplements are required. No need for organic growth factors could be demonstrated under the conditions of cultivation used. Sea water, especially as it concentrates by evaporation, probably contains most of the required trace elements in sufficient quantity. It is possible that for mass cultivation of very dense algal suspensions, supplements of the micronutrients will be required.

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